Identification and physiological evaluation of the components from *Citrus* fruits as potential drugs for anti-corpulence and anticancer

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Abstract— On the basis of monitoring the prevention of accumulation of lipid droplets in mouse 3T3-L1 preadipocyte cells and inhibition of the proliferation of human colon cancer HT-29 cells, effective anti-corpulence and anticancer compounds were isolated from the peel of *Citrus* fruits. These bioactive components were identified as polymethoxyflavones and coumarin derivatives by spectroscopic analyses. 5-hydroxy-6,7,8,3',4'-pentamethoxyflavone had the greatest anti-corpulence effects and 3,5,6,7,8,3',5'-heptamethoxyflavone had the greatest anticancer effects. Furthermore, distributions of those bioactive components in the peel of 10 species of *Citrus* fruits were demonstrated by HPLC analyses.

Introduction

Citrus fruits are known to be an abundant resource of compounds that might help prevent lifestyle-related diseases such as diabetes, high blood pressure and cancer [1-15]. In recent years, many studies have focused on the anticancer properties of *citrus* components, especially polymethoxyflavones and their glycosides [3,5-8,12]. However, little is known about active components with anti-corpulence properties [13]. As a part of our investigation of natural products with beneficial properties, we investigated the compounds with anti-corpulence and anticancer activities in *citrus* fruits. This paper considers the isolation and structure determination of the bioactive constituents with anti-corpulence and anticancer activities from the peel of *citrus* fruits, such as *C. tachibana*, *C. kinokuni*, and *C. canaliculata*. Distributions of the components with anti-corpulence and anticancer activities in the peel of several *citrus* species were also examined.

Results and Discussion

The mature fruits of 17 species of *Citrus* listed in Table 1 were collected from the "Citrus Park" of Onomichi-city, Hiroshima Prefecture, Japan during December to February. The peel and juice of the *citrus* fruits were separated. They were extracted with ethanol (EtOH).

Table 1. *Citrus* species investigated for bioactive components

Ref. (#)	Scientific name	Common name
1	*C. latifolia* Tanaka	Lime
2	*C. limonimedica Lush*	Busyukan
3	*C. limon* (L.) Burm.	Lemon
4	*C. maxima* (Burm) Merr.	Anseikan
5	*C. hassaku* hort. ex Tanaka	Hassaku
6	*C. natsudaidai* Hayata	Natsudaidai
7	*C. canaliculata* hort. ex Yu.Tanaka	Kikudaidai
8	*C. unshiu Marcow* x *C. sinensis Osbeck*	Kiyomi
9	*C. sinensis* Osbeck *var. brasiliensis* Tanaka	Navel
10	*C. yuge-hyoukan* hort. ex Tanaka	Yugehyoukan
11	Chance seedling of *C. tamurana* hort. ex Tanaka	Haruka
12	*C. unshiu* Marcow	Unshu
13	*C. junos* Siebold ex Tanaka	Tadanishiki
14	*C. kinokuni* hort. ex Tanaka	Kishu
15	*C. tachibana* (Mak.) Tanaka	Tachibana
16	*C. leiocarpa* hort. ex Tanaka	Koji
17	*Fortunella margarita* (Lour.) Swingle	Kinkan

In the routine stage of screening, these crude EtOH extracts were tested for inhibitory effects against the accumulation of lipid droplets in mouse 3T3-L1 preadipocyte cells, and against the viability of the human colon cancer cell line HT-29. As shown in Figs 1 and 2, significant activity was seen in the peel extracts compared with the juice extracts. In particular, strong anti-corpulence activities were shown in the peel extracts of *C. limon* (Ref. #3 in Table 1), seedling sp. of *C. tamurana* (#11), *C. kinokuni* (Ref. #14), *C. tachibana* (#15) and *C. leiocarpa* (#16), and strong anticancer activitied were shown in *C. canaliculata* (#7), seedling sp. of *C. tamurana* (#11), *C. kinokuni* (#14) and *C. tachibana* (#15).

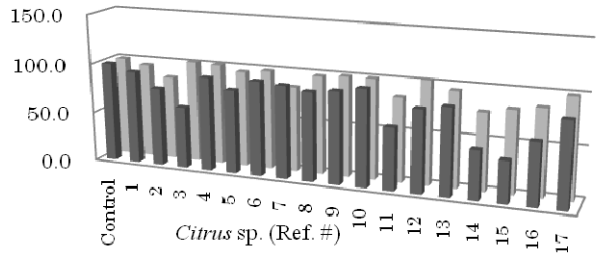


Fig. 1. Anti-corpulence activity of EtOH extracts of *citrus* fruits. Black stick: peel extracts, Silver stick: juice vesicle extracts.

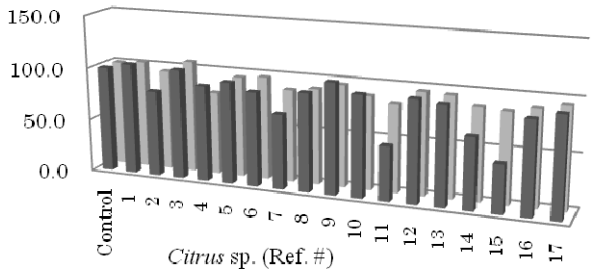


Fig. 2. Anticancer activity of EtOH extracts of *citrus* fruits. Black stick: peel extracts, Silver stick: juice vesicle extracts.

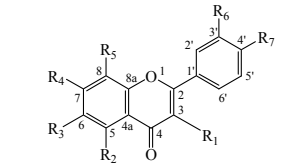
Therefore, the peel extracts of *C. canaliculata,* seedling sp. of *C. tamurana*, *C. kinokuni* and *C. tachibana* were further investigated. These EtOH extracts were partitioned between ethyl acetate (EtOAc) and water. The bioassay showed that the EtOAc-soluble fraction had stronger anti-corpulence and anticancer activities compared with the water-soluble fraction, as shown in Table 2.

Table 2. Anti-corpulence and anticancer activities of the ethyl acetate- and water-soluble fractions

Citrus sp. (Ref. #)	Fraction	Anti-corpulence IC_{50} (μg/ml) [a]	Anticancer IC_{50} (μg/ml) [b]
C. canaliculata (#7)	EtOAc	--	57
	H_2O	--	750
Seedling sp. of *C. tamurana* (#11)	EtOAc	--	31
	H_2O	--	550
C. kinokuni (#14)	EtOAc	50	45
	H_2O	>500	850
C. tachibana (#15)	EtOAc	50	45
	H_2O	>500	800

[a] IC_{50} denotes the concentration that inhibits the accumulation of lipid droplets by 50% of the control level. [b] IC_{50} denotes the concentration that inhibits cell growth by 50% of the control experiment.

Next, on the basis of the assays for anti-corpulence and anticancer activities, the EtOAc-soluble fractions were further separated by column chromatography on silica gel and HPLC on ODS column to generate ten compounds (**1**–**10**). Compounds **1**–**6** have strong anti-corpulence and anticancer activities, and compounds **7** and **8** show only anticancer activity. Compounds **1**–**6** were mainly isolated from the EtOAc-soluble fractions of seedling sp. of *C. tamurana, C. tachibana* and *C. kinokuni*, and compounds **7**–**10** were isolated from *C. canaliculata*.



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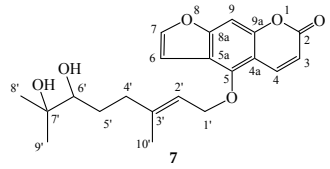
3: R_1=R_2=R_3=R_4=R_5=R_6=R_7=OMe
4: R_1=H, R_2=R_3=R_4=R_5=R_6=R_7=OMe
5: R_1=R_5=H, R_2=R_3=R_4=R_6=R_7=OMe
6: R_1=R_3=H, R_2=R_4=R_5=R_6=R_7=OMe



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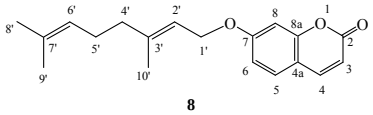

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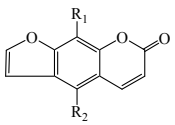
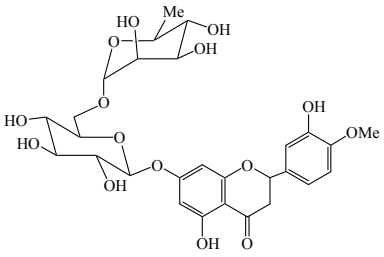


9: R_1=H, R_2=OH
10: R_1=OH, R_2=H



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The structures of the compounds were identified by analysis of the ^{1}H- and ^{13}C-NMR and FABMS spectra. Compounds **1**–**6** were known polymethoxyflavones,

namely 5-hydroxy-6,7,8,3',4'-pentamethoxyflavone (**1**) [9,16], 5,6,7,8,4'-pentamethoxyflavone (**2**) (Tangeritin) [9,16,17], 3,5,6,7,8,3',4'-heptamethoxyflavone (**3**) [7,17], 5,6,7,8,3',4'-hexamethoxyflavone (**4**) (Nobiletin) [9,16,17], 5,6,7,3',4'-pentamethoxyflavone (**5**) (Sinensetin) [9,16,17] and 5,7,8,3',4'-pentamethoxyflavone (**6**) [9,17]. Compounds **7**–**10** were known coumarin derivatives, namely 6',7'-dihydroxybergamottin (**7**) [4,20], 7-geranyloxycoumarin (**8**) (auraptene) [4,20], 5-hydroxypsoralen (**9**) [4], and 8-hydroxypsoralen (**10**) [4].

The biological activities of the purified compounds **1**–**10** were assessed and inhibition of lipid droplet accumulation in mouse 3T3-L1 preadipocyte cells (anti-corpulence activity) and inhibition of proliferation of human colon cancer HT-29 cells (anticancer activity) were measured using hesperidin (**11**) as a reference compound for biological activity. The IC_{50} values for these inhibition effects are shown in Table 3. Polymethoxyflavones, compounds **1**–**6**, exhibited significantly high anti-corpulence and anticancer activities, whereas coumarin derivatives, compounds **7** and **8**, showed only anticancer activity. 5-hydroxy-6,7,8,3',4'-pentamethoxyflavone (**1**) had the greatest anti-corpulence activity and 3,5,6,7,8,3',4'-heptamethoxyflavone (**3**) had the greatest anticancer activity. In addition, none of the tested compounds had cytotoxic effects on the tested cells, as evident by no changes in the numbers of viable cells after 1 day of treatment.

Table 3. Anti-corpulence and anticancer activities of the components of *Citrus* sp.

Compound	Anti-copulence IC_{50} (μM) [a]	Anticancer IC_{50} (μM) [b]
1	12	57
2	77	48
3	30	28
4	24	50
5	67	59
6	28	43
7	>500	52
8	>500	47
9	>500	>500
10	>500	>500
11	142	311

[a] IC_{50} denotes the concentration that inhibits accumulation of lipid droplets by 50% of the control experiment. [b] IC_{50} denotes the concentration that inhibits cell growth by 50% of the control experiment.

Distribution of these bioactive flavones and coumarins in the peels of ten species of *citrus* fruits was analyzed by HPLC. As shown in Table 4, the polymethoxyflavones, compounds **1**–**6**, were abundant in the peels of Chance seedling of *C. amurana* (#11), *C. kinokuni* (#14), *C. tachibana* (#15) and *C. leiocarpa* (#16), whereas coumarin derivatives, compounds **7** and **10**, were in those of *C. hassaku* (#5) and *C. canaliculata* (#7). However, contents of polymethoxyflavones **1**–**6** in the peels of *C. limon* (#3) were very low, although the peel extract had the strong anti-corpulence activity, as shown in Fig. 1. This might mean that the peels of *C. limon* contain the other active components.

Experimental

General experimental procedures

Analytical and preparative TLCs were carried out on glass sheets (0.25 mm and 0.5 mm) coated with silica gel (Merck silica gel 60; GF 254) and column chromatography was performed on silica gel column (Merck silica gel 60; 230-400 mesh) using a gradient solvent of 0–50% EtOAc in hexane. HPLC assays were performed using an ODS-3 column (3 mm x 25 cm) equipped with a UV detector at 254 nm and using a gradient solvent of 50–70% MeOH in H_2O. ^{1}H-NMR (500 MHz), ^{13}C-NMR (125 MHz), and 2D-NMR spectra were recorded using a JEOL LA500 spectrometer. Fast Atom Bombardment Mass Spectroscopy (FABMS) was measured on a JEOL SX102A spectrometer.

Cell cultures

Human colon cancer HT-29 cells, mouse 3T3-L1 preadipocyte cells, and human embryonic kidney (HEK) 293 cells were maintained in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (maintenance medium) at 37°C in 5% CO_2.

Materials

The 17 species of *citrus* fruits (Table 1) were cultivated and collected at the Citrus Park in Setoda-cyo, Onomichi-city, Hiroshima Prefecture, Japan, during the period December to February of 2005–2007. Hesperidin (**11**) was purchased from Sigma Chemical Co., Ltd.

Extraction and isolation

Fresh fruits were separated into the peel and juice vesicle parts. These were homogenized using a blender, soaked in ethanol (EtOH), and then extracted by 1-month treatment under dark and cool conditions. After filtration, each extract was concentrated *in vacuo* and these extracts were analyzed by bioassays (the anti-corpulence with 3T3-L1 preadipocytes mouse cells and the anticancer with human colon cancer HT-29 cells), as shown in Figures 1 and 2. Among the extracts that showed good activities, the peel extracts of *C. canaliculata* (*#7),* and seedling sp. of *C. tamurana* (#11), *C. kinokuni* (#14) and *C. tachibana* (#15) were further separated into ethyl acetate (EtOAc)- and water-soluble fractions.

Table 4. Distribution of biologically active flavones (**1**–**6**) and coumarins (**7** and **8**) in the peels of *citrus* fruits

Citrus sp. (Ref. #)	EtOAc ext.	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
C. limonimedica Lush (#2)	215	0.3	0.0	0.0	0.0	0.4	0.0	tr.	4.1
C. limon (L.) Burm. (#3)	370	0.0	0.0	tr.	0.0	0.0	0.0	tr.	5.2
C. hassaku hort. ex Tanaka (#5)	510	0.7	1.8	4.5	3.6	tr.	tr.	tr.	7.7
C. canaliculata hort. ex Yu.Tanaka (#7)	495	1.6	tr.	tr.	tr.	tr.	tr.	7.9	1.0
C. sinensis Osbeck *var. brasiliensis* Tanaka (#9)	330	1.2	3.2	8.7	28.5	14.3	1.2	tr.	tr.
Chance seedling of *C. tamurana* hort. ex. Tanaka (#11)	348	tr.	7.2	31.2	23.7	2.6	1.6	tr.	tr.
C. unshiu Marcow (#12)	98	0.1	1.6	10.6	6.5	1.6	0.9	tr.	tr.
C. kinokuni hort. ex Tanaka (#14)	346	11.9	44.3	3.2	69.2	17.3	8.3	0.5	tr.
C. tachibana (Mak.) Tan. (#15)	541	18.5	53.2	10.0	111.1	32.5	20.6	0.4	tr.
C. leiocarpa hort. ex Tanaka (#16)	396	19.0	32.4	1.6	71.9	11.3	7.4	1.3	tr.

Each value is the average of two replications (mg/100 g flesh weight).

By monitoring the bioactivity, each of the EtOAc-soluble fractions was separated by column chromatography on silica gel using EtOAc–hexane to generate several components such as **1** (31 mg), **2** (121 mg), **3** (9 mg), **4** (190 mg), **5** (49 mg), **6** (22 mg), **7** (1.0 mg) from the EtOAc extract (500 mg) of *C. kinokuni* peels (150 g), and **7** (19 mg), **8** (3 mg), **9** (1.0 mg), **10** (1.0 mg) from the EtOAc extract (1.7 g) of *C. canaliculata* peels (350 g). The structures of the compounds **1**–**10** were identified by the spectroscopic analyses with the ^{1}H- and ^{13}C-NMR and FABMS and comparison of these data with reported data [4,7,9,16,17,20]. These compounds were further purified by preparative HPLC with an ODS-3 column using 50–70% MeOH in H_2O, and used for final bioassays (Table 3).

The distribution of the bioactive compounds **1**–**8** in the EtOAc extracts from the peel of several *citrus* fruits were analyzed by HPLC with an ODS-3 column using 50–70% MeOH in H_2O. The results are summarized in Table 4.

Assay of adiposity differentiation.

For differentiation assays, confluent 3T3-L1 cells were cultured in differentiation media (maintenance media plus 0.5 mM 3-isobutyl-1-methylxanthine [IBMX], 5 µg/ml insulin and 1 µM dexamethasone [DEX]) for 2 days. Then, differentiation media was replaced with adipocyte growth media (maintenance media supplemented with 5 µg/ml insulin) with or without each compound, which was changed every 2 days. Differentiated 3T3-L1 cells were fixed with 4% buffered paraformaldehyde for 15 min. A stock solution of 0.3% Oil-Red-O (Sigma) in isopropanol (w/v) was diluted 6:4 prepared in water, filtered, and added to fixed cells. Cells were washed twice in phosphate-buffered saline (PBS) and photographed. The cells were solubilized with 1% Nonidet P-40 (Sigma) in isopropanol, and quantified by measuring the absorbance at 520 nm. The assays were carried out five times for each sample and the data are expressed to the mean percentage of lipid droplet accumulation as compared to the respective control experiment. A statistical deviation was less than 10%.

Anticancer assay.

HT-29 cells (human colon cancer cell line, American Type Culture Collection) were routinely cultured in Dulbecco's modified Eagle's medium-high glucose (Sigma) containing 10% bovine calf serum in a 5% CO_2 humidified environment at 37°C. 50 units/ml penicillin and 50 µg/ml streptomycin were added to the media. The cells were plated at a density of 8,000 cells/well on 96-well plates in 100 µl of fresh media and assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. 24 h after plating, the cell media was exchanged with 100 µl of dimethylsulfoxide (Me_2SO) solution containing 10% bovine calf serum and the indicated compound. After 72 h cultivation, 10 µl of MTT 5 mg/ml was added to each well and incubated for a further 4 h. The formazan crystals resulting from mitochondrial enzymatic activity of the MTT substrate were solubilized with Me_2SO. Absorbance was measured at 550 nm using a microplate reader (TFB). Cell number was expressed as absorbance relative to that of untreated controls. The assays were carried out ten times for each sample and the data are expressed to the mean percentage of viable

cells as compared to the respective control experiment. A statistical deviation was less than 10%.

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